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Year: 2007

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## **Interrelation of directly measured oxygenation levels, erythropoietin and erythropoietin receptor expression in spontaneous canine tumours**

Rohrer Bley, Carla ; Wergin, Melanie ; Roos, Malgorzata ; Grenacher, Beat ; Kaser-Hotz, Barbara

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DOI: <https://doi.org/10.1016/j.ejca.2006.12.007>

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ZORA URL: <https://doi.org/10.5167/uzh-23998>

Journal Article

Accepted Version

Originally published at:

Rohrer Bley, Carla; Wergin, Melanie; Roos, Malgorzata; Grenacher, Beat; Kaser-Hotz, Barbara (2007). Interrelation of directly measured oxygenation levels, erythropoietin and erythropoietin receptor expression in spontaneous canine tumours. *European Journal of Cancer*, 43(5):963-967.

DOI: <https://doi.org/10.1016/j.ejca.2006.12.007>

**Interrelation of Directly Measured Oxygenation Levels, Erythropoietin and Erythropoietin  
Receptor Expression in Spontaneous Canine Tumours**

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Running title: Oxygenation, Epo and EpoR Expression in Canine Tumours

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## Abstract

The expression of the hypoxia-inducible protein erythropoietin in tumour cells correlates with levels of tumour hypoxia. Our aim was to look for an interrelation of *directly* measured oxygenation levels, the presence of tissue erythropoietin and its receptor. Data of tumour oxygenation status, plasma and tissue erythropoietin and its receptor in a group of spontaneously occurring tumours in 15 dogs were collected. Polarographic tumour oxygen partial pressure measurements were obtained and data were correlated.

Significant positive correlations were found between tissue erythropoietin and the percentages of pO<sub>2</sub> values  $\leq 10$  mmHg. Multivariate analysis revealed no parameters influencing plasma erythropoietin levels.

Our results show that a co-expression of erythropoietin receptor and its ligand in spontaneous canine tumours exists, that the level of hypoxia in tumour cells correlates with the level of tissue erythropoietin and suggest the need to be quantitatively and functionally tested as novel prognostic biological parameters in neoplastic tissues.

**Key words:** tumour hypoxia, erythropoietin, erythropoietin receptor

## Introduction

It has been shown, that Epo<sup>1</sup> and EpoR are not only produced and expressed by various non-erythroid tissues, but also by human malignancies (1-3). As a pleiotropic cytokine, Epo on one hand acts as a haematopoietic growth factor, but it also exerts proangiogenic and tissue-protective effects in other organs. Activation of Epo-EpoR signalling pathways in cancer cells may be followed by modulation of tumour environment such as angiogenesis, increased proliferation and changes in apoptotic ability (4). Furthermore response to treatment such as chemoradiation may be influenced. Tumour hypoxia, a factor associated with tumour aggressiveness, treatment resistance and poor prognosis itself (5-7), seems to be one of the up-regulators and modulators of Epo production as a direct result of hypoxia inducible factor-1 activation (4, 8). It has been shown in vivo and in vitro that Epo and EpoR expression can be partially colocalized with tumour tissue hypoxia (3, 9). However, these studies were assessing the relationship of tumour hypoxia and the expression of EpoR and its ligand in a semiquantitative way. In the present study, we hypothesized that hypoxic tumours have high tissue Epo expression and that this relationship can be directly shown by correlating invasive polarographic oxygen measurements with quantitative analyses of tumour tissue Epo.

## Materials and Methods

**Patients.** Fifteen canine patients with tumours of various histologies, enrolled in a tumour hypoxia study approved by the Swiss Veterinary authorities were included in this study. The primary tumours were localized in the oral cavity in 11 patients, on the extremities and skull in two patients each. Two or more quick frozen tumour tissue specimens were available from each patient. The site, histological diagnosis and tumour volumes are listed in Table 1.

**Measurement of tumour hypoxia.** Tumour oxygen partial pressure measurements were performed as previously described in dogs (10), using an pO<sub>2</sub>-Histogram (Helzel Medical Systems, Kaltenkirchen, Germany). The needle electrode was placed within the tumour tissue under ultrasound guidance (ATL 5000, Philips Medical Systems, Zurich, Switzerland). At least three different electrode tracks and a minimum of 50 recorded values were acquired for reliable statistical analysis (11).

Oxygenation status of individual tumours was described using the median  $pO_2$  and the hypoxic fractions (% of  $pO_2$  values  $\leq 10$  mmHg,  $\leq 5$  mmHg and  $\leq 2.5$  mmHg, respectively). Tumour size was measured with callipers and the volume was calculated based on the formula:  $\pi/6 \times \text{height} \times \text{width} \times \text{depth}$ , which approximately describes the volume of an ellipsoid. The hypoxic subvolume (HSV) was calculated by the formula: volume (ccm)  $\times$  hypoxic fraction (% of  $pO_2$ -values  $\leq 5$  mmHg).

**Sample preparation.** Tumour biopsies were taken immediately after the oxygen measurements, right before the start of radiation therapy and the samples were quick frozen in liquid nitrogen. The samples were stored at  $-80^\circ\text{C}$  until further use. For protein extraction the specimens were transferred directly from  $-80^\circ\text{C}$  in 1ml of ice cold homogenization buffer (0.27 M Sucrose, 2 mM EDTA pH 8.0, 1 % NP-40, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin, 1 mM NaVa) in a 5 cm<sup>2</sup> dounce homogenizer on ice: tissue was dounced 25 times with a pestle, and then transferred on a sucrose cushion. Tissue lysate was centrifuged for 10 min at  $4^\circ\text{C}$  at 3000 rpm and protein concentration was measured. For analysis of plasma Epo three millilitres of blood were collected into sterile CTAD tubes (Beckton and Dickinson Vacutainer System, France) and placed on ice. The tubes were centrifuged within 15 min at  $2500 \times g$  for 30 min at  $4^\circ\text{C}$ . The resulting plasma was separated and stored immediately at  $-80^\circ\text{C}$ .

**RIA.** Plasma and tissue erythropoietin was measured using a commercial kit (Epo-Trac<sup>TM</sup> <sup>125</sup>I RIA kit, DiaSorin, USA) applying the method previously described by Glaus et al. (12). Tissue Epo was measured in normal tissues in order to establish control values in a series of canine tissues, such as lymphnode, liver, muscle and uterus.

**Western blotting.** Epo receptor status of analysed tumour specimens was performed by immunoblotting technique. Therefore, protein samples (100  $\mu\text{g}$ /well) were resolved by denaturing electrophoresis on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membrane was blocked for 2 h in 4% nonfat dry milk in PBS, 0.5% Tween 20, rinsed, and subsequently incubated with the EpoR antibody (H-194, Santa Cruz)

101 diluted in PBS and 1.0% Tween 20 was diluted 1:1000 over night at 4°C. The membrane was washed  
102 with PBS, 1.0% Tween 20 and incubated with the secondary antibody (horseradish peroxidase-  
103 conjugated goat anti-rabbit antibody, Amersham Pharmacia Biotech) diluted 1:5000 in 0.5% Tween 20  
104 in PBS. After washing the membrane three times, the protein was detected using enhanced  
105 chemiluminescence (ECL, Amersham Pharmacia Biotech).

106  
107 **Statistical analyses.** Description of patient data is given by mean ( $\pm$  SD) unless otherwise  
108 specified. The dependence of different tumour and patient characteristics on plasma and tissue Epo  
109 levels was evaluated by correlation (Wilcoxon rank test, Fisher's exact probability test). Univariate  
110 proportional hazards and multiple Cox-regression analysis were used for further testing of influences  
111 of any of the descriptors on Epo status. Distribution in HSV and tumour volumes were skewed, thus  
112 logarithmically transformed values were used rather than raw measurements. In all calculations p-  
113 value of  $< 0.05$  were considered significant. For statistical analysis StatView 5.0.1 was used.

## 114 115 116 **Results**

117 **Plasma Epo and haematological parameters.** Mean plasma erythropoietin levels were within  
118 normal limits with a mean of 18.8 mU/ml (range 13.6-27.2 mU/ml, normal: 18 mU/ml (range 0-36  
119 mU/ml)). The haematological parameters were within normal limits with mean haematocrit of 43.5%  
120 (range: 35.5-54.0%, normal: 37-55%), haemoglobin of 15.2 g/dl (range: 12.3-10.0 g/dl, normal: 12-18  
121 g/dl). No difference could be found for any of the parameters with respect to the histological groups.

122  
123 **Tumour hypoxia.** The oxygen measurements were comparable to previous findings (13). More  
124 than 20% of the values were below 10mmHg, 16% of all readings were below 5, 13% of all readings  
125 below 2.5 mmHg, indicating severe hypoxia. The mean of all median pO<sub>2</sub> values was 27 mmHg  
126 (range: 0-95 mm Hg).

**Tissue Epo and EpoR expression.** The control values of normal canine tissue Epo expression was established and is presented in Table 1.

All of the examined tumour tissues were positive for tissue Epo (n=15), and 93% of these also expressed erythropoietin receptor. Mean tissue Epo levels were 20.0 mU/ml (range: 0.8-65.8 mU/ml, normal: 0.7-2.7 mU/ml (Table 1).

**Relationship between tumour hypoxia and Epo expression.** Significant positive correlations were found between tissue Epo and the percentages of pO<sub>2</sub> values ≤ 10 mmHg (r<sup>2</sup>=0.93, 95% CI (0.79, 0.98) p=<0.0001), as well as the hypoxic subvolume (r<sup>2</sup>=0.86, 95% CI (0.61, 0.95), p=<0.0001). Tumours with median pO<sub>2</sub> values ≤ 10 mmHg had significantly higher tissue erythropoietin levels (r<sup>2</sup>= -0.65, 95% CI (-0.87, -0.21) p=0.007) than tumours with median pO<sub>2</sub> values >10 mmHg.

No correlations between the percentages of pO<sub>2</sub> values ≤ 10 mmHg, the median pO<sub>2</sub>, or the level of tissue Epo and plasma erythropoietin concentrations were found.

Multivariate analysis revealed that neither of the haematological parameters nor tumour volumes influenced the amount of expression of tissue or plasma Epo levels (p>0.27), while the influence of the median pO<sub>2</sub> and the pO<sub>2</sub> values ≤ 10 mmHg again were found to be important (p<0.03).

## **Discussion**

Our findings have strong implications for theories regarding Epo biology in spontaneous tumours. The level of tissue Epo but not plasma Epo expression strongly correlates with the level of tumour hypoxia, indicating a paracrine role of tissue Epo at the cellular level in the tumour (14). The fact, that stronger correlation concerning the percentages of low pO<sub>2</sub> values and tissue Epo, rather than the median pO<sub>2</sub> value was found, indicates a preferential induction of tissue Epo at very low oxygen levels. This finding is further supported by the preferential distribution of Epo staining in perinecrotic (highly hypoxic) regions (15). Furthermore, Arcasoy and colleagues (2005) describe a significant

positive correlation between of regional tissue Epo expression and the hypoxia-marker pimonidazole, which strongly supports the concomitance of high Epo expression in tissue regions with low pO<sub>2</sub> (3). While polarographically measurable low oxygen tension in tumours are known to modulate the sensitivity of cancer cells to various treatment modalities (5, 7, 16), the effect of hypoxia-induced endogenous Epo on treatment outcome has not yet been described.

Intermittent hypoxia is an effective stimulus for Epo synthesis and at high altitudes physiological plasma Epo rises rapidly, peaking at 20-48 hours, thereafter declining and reaching normal level values again (12, 17).

The fact that in this study plasma erythropoietin levels did not correlate with tumour hypoxia, may indicate that in a chronic condition as exerted by a neoplastic disease, either the normal values of plasma Epo have already been re-established, and/or most of the tissue erythropoietin produced by the tumour directly binds to its local receptors and the small amounts released into the blood stream do not influence plasma erythropoietin levels. This finding, together with the coexpression of Epo and EpoR in tumour cells, is indicative of an autocrine-paracrine activation loop, presenting a potential therapeutic target in tumours where the Epo/EpoR signalling may be involved in tumour progression and angiogenesis (4).

Similar to findings of other studies (3, 9, 15), 93% of all evaluated tumour tissues in this study were positive for EpoR. In vitro, hypoxia induces nuclear accumulation of the hypoxia inducible factor (HIF-1) protein and also upregulates EpoR-protein expression (1, 8, 15), and Molhyedin et al. found in their study in HNSCC cell lines a hypoxia-inducible upregulation of EpoR rather than tissue Epo (15). However, in this and other studies the expression of Epo-R is also found in normoxic tumours, indicating the induction through another oncogenic mechanism (8).

Epo must act through binding of EpoR which will in turn stimulate downstream signalling in the cell through the JAK/STAT pathway (9). However, the presence of EpoR does not guarantee its functional capacity (9) and the Western-blot analysis does not discriminate between EpoR expression localized to



the cell surface and expression localized to the cytoplasmic region of the cell. Although cellular proliferation and anti-apoptotic protection at suprapharmacologic concentrations of Epo has been shown in irradiated tumour cells in vitro (15, 18), the cellular signalling mechanism must yet be proven. The presence of a functional EpoR/Epo-system, however, may contribute to the selection of cells with diminished apoptotic potential and relative resistance to various cancer treatments (8, 18, 19), and may be further enhanced by exogenous Epo administration. These in vitro findings and the high percentage of Epo in various canine tumour tissues offer support for the prior raised hypothesis of a negative effect of administered rHuEpo to anaemic tumour patients (20).

In conclusion we have demonstrated that there is a strong direct correlation between the prognostic significant polarographically measured amount of hypoxia (21) and the expression of Epo in canine malignant tissue. Since increased Epo signalling may be one of the mechanisms by which hypoxia promotes tumour aggression, the presence of Epo and EpoR need to be quantitatively and functionally tested as novel prognostic biological parameters in neoplastic tissues.

<sup>1</sup>The abbreviations used are: Epo: erythropoietin; EpoR: erythropoietin receptor; pO<sub>2</sub>: partial pressure oxygen tension

**Conflict of interest statement: the authors have no relationship, financial or otherwise, with any manufacturers or distributors of products evaluated in the paper.**

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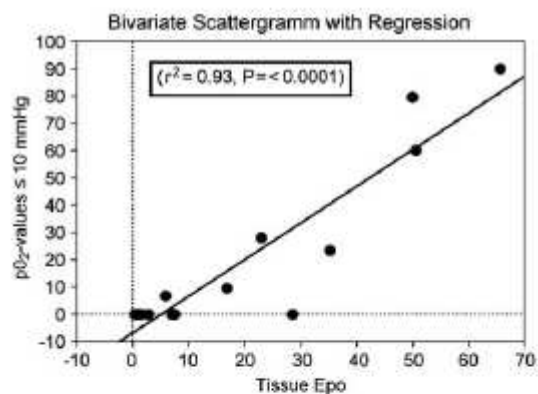
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## Figure Legends

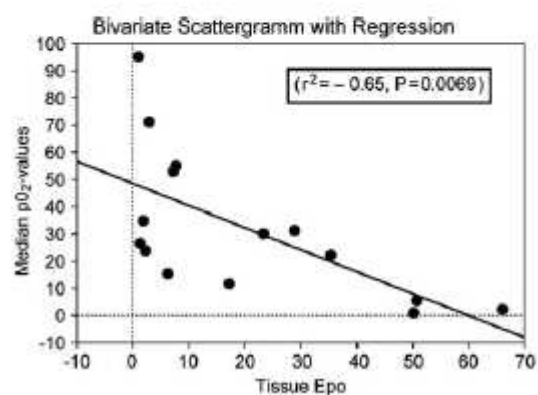
Figure 1: Correlations between tissue Epo and the percentages of  $pO_2$  values  $\leq 10$  mmHg: the more values of low oxygen tension are present, the higher the tissue Epo expression

Figure 2: Correlations between tissue Epo and the median  $pO_2$ : the lower the median oxygen pressure, the higher the tissue Epo expression

Table 1: Descriptive tumour characteristics



**Fig. 1 – Correlations between tissue Epo and the percentages of  $pO_2$  values  $\leq 10$  mmHg: the more values of low oxygen tension are present, the higher is the tissue Epo expression.**



**Fig. 2 – Correlations between tissue Epo and the median  $pO_2$ : the lower the median oxygen pressure, the higher the tissue Epo expression.**

Patient	Histology	Location	Volume (ccm)	Hypoxic subvolume (ccm)	Median pO <sub>2</sub> (mmHg)	Plasma Epo (mU/ml)	Tissue Epo (mU/ml)	Epo receptor
1	Fibrosarcoma	Oral cavity	50.3	24.7	5	13.6	50.5	+
2	Squamous cell carcinoma	Oral cavity	13.1	0	31	19.4	28.7	+
3	Squamous cell carcinoma	Oral cavity	9.2	0.5	22	15.0	35.4	+
4	Hemangiopericytoma	Limb	100.5	23.3	30	27.3	23.2	+
5	Fibrosarcoma	Skull	149.7	126.7	2	13.8	65.8	+
6	Malignant melanoma	Oral cavity	26.4	20.1	1	17.3	50.0	-
7	Malignant melanoma	Oral cavity	15.7	0	55	26.7	7.6	+
8	Squamous cell carcinoma	Oral cavity	0.9	0	32	18.5	1.7	+
9	Squamous cell carcinoma	Oral cavity	45.2	0	15	15.4	6.2	+
10	Fibrosarcoma	Oral cavity	13.2	0.13	11	19.8	17.0	+
11	Hemangiopericytoma	Limb	206.5	0	52	14.3	7.0	+
12	Histiocytic sarcoma	Skull	67.8	0	71	18.3	2.8	+
13	Malignant melanoma	Oral cavity	7.6	0	95	21.7	0.8	+
14	Malignant melanoma	Oral cavity	16.2	0	26	20.3	1.2	+
15	Malignant melanoma	Oral cavity	29.9	0	23	15.3	2.0	+
Normal tissue values (median 1.8)		Lymphnode					2.6	
		Liver					1.2	
		Muscle					0.7	
		Uterus					2.7	